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The *Acanthamoeba* spp. in Water Sources from Zanjan Province, Northwest of Iran

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ABSTRACT

Background: The genus of *Acanthamoeba* is an opportunistic protozoan parasite with a worldwide distribution where it is able to cause fatal granulomatous amoebic encephalitis (GAE) and amoebic keratitis (AK). This organism inhabits in the wide range of natural and man-made aquatic environments. The present study was carried out to evaluate the presence of *Acanthamoeba* spp. in the various water resources of Zanjan province, northwest Iran, using both morphological and molecular methods.
Methods: The Water samples were randomly collected from 30 water sources in different parts of Zanjan, Iran, between April 2015 and May 2016. Then, the samples were cultured on non-nutrient agar and the *Acanthamoeba* genus identified by morphological characters. The polymerase chain reaction (PCR) was performed using the 18S rRNA gene as a molecular marker.
Results: The obtained data showed that, out of the 60 water samples collected, 30 (50%) were positive for *Acanthamoeba* spp. According to morphological and molecular approaches.
Conclusion: The present investigation is the first report of the distribution of *Acanthamoeba* spp. in the various water sources of Zanjan province, gives baseline knowledge regarding water contamination with *Acanthamoeba* spp. in these areas and emphasizes the necessity of more attention to water sources in order to prevent infections associated with *Acanthamoeba* spp.

1. Introduction

The genus of *Acanthamoeba* is a free living amoeba which is an important opportunistic parasite with a cosmopolitan distribution [1, 2].

This amphizoic organism has been isolated from air, soil, dust, sewage, sediments, contact lenses as well as the clinical specimens and is particularly plentiful in water [3, 4]. The genus of *Acanthamoeba* has two developmental stages in its life cycle trophozoite and cyst, [5]. The trophozoite, is an active form, which the genus of *Acanthamoeba* is able to feed, grow, move and reproduce, and the cyst, is a non-active form. The double-layered coat cyst of this amoeba is resistant to disinfectants, antibiotics, UV radiation, and the chemical agents. Furthermore, it can survive as

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well at -2 ˚ C to 45 ˚ C [6-9]. The pathogenic genotypes of *Acanthamoeba* are the etiological agents of granulomatous amoebic encephalitis (GAE), skin, lung, kidney, liver, spleen, prostate and uterus infections in the immunocompromised individuals.

Also some strains can cause amoebic keratitis in the healthy people who are soft contact lens users or have a history of corneal injury [10-13].

Moreover, the *Acanthamoeba* can carry and transmit a large number of microorganisms including the Listeria monocytogenes, the Echerichia coli serotype O157, Burkholderia cepacia, Vibrio Cholerae, Psudomonas aeruginosa, Helicobacter pylori, Chlamydophila pneumonia, Coxiella burnetii, Legionella spp, Mycobacterium spp, Coxsackie virus, Echovirus and Adenovirus [9, 14-16]. Thus, the *Acanthamoeba* plays both direct and indirect harmful roles in human's health [17, 18].

During the past years, the *Acanthamoeba* keratitis cases are regularly reported in the medical centers exist in Iran [19]. Moreover, some researchers have showed the presence of the undistinguished encephalitis in immunocompromised patients in the country [20].

The studies have demonstrated that the aquatic environments are among the most important sources for the transmission of *Acanthamoeba* spp to human's body [7, 13, 21]. The occurrence of the *Acanthamoeba* genus in aquatic sources has been determined in different parts of Iran [13, 17, 22-25], but there is no information from Zanjan province. The present study was designed to detect the presence of Acanthamoeba genus in Zanjan province, northwest of Iran in the surface waters using a combined culture assay and the PCR method.

2. Materials and Methods

2.1. Sample collection

This descriptive cross-sectional survey was carried out in Zanjan province (36 ˚ 40´ N and 48 ˚ 29´ E), in northwest of Iran, between April 2015 and May 2016. Overall, 60 surface water samples were collected from 30 various sources including the ponds in parks, the squares, the fountains in green fields dams and channels. From each sampling point, two water samples were placed in 500mL labeled and the sterile bottles transported immediately to the laboratory in the department of parasitology and Mycology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran, for further procedures.

2.2. Isolation and Cultivation

Each sample was filtrated through a 0.45µm pore-size cellulose nitrate membranes using a vacuum pump [26]. After that, the filters were put on to the 1.5% non-nutrient agar (NNA) plates enriched with heat-killed Escherichia coli as a food source for the outgrowth of amoebae [27, 28]. The plates were incubated at the room temperature and monitored daily for up to 2 month in order to evaluate the growth of *Acanthamoeba* using an inverted microscope [29]. Then all the positive cultures were cloned to obtain a single cell line as well as to eliminate the fungal and bacterial contamination [30].

2.3. The Morphological identification

The preparations were tested for the presence of trophozoites and the cysts using the Giemsa staining assay [31] according to pages of taxonomy keys [29].

2.4. The DNA extraction

The growing amoebae were harvested from the surface of culture plates, washed with the sterile PBS, and centrifuged at 1000 rpm for 15mins. The DNA extraction was performed using the QIA amp DNA mini kit (Qiagene GmbH, Germany) according to the manufacturer's instructions. Then, the DNA was kept at -20˚C until the amplification of polymerase chain reaction (PCR).

2.5. PCR

The PCR procedure was carried out by amplifying a 423bp to 551bp region of the 18S rRNA gene (Rns) defined as ASA. The S1 that includes the hyper variable diagnostic fragments 3

(DF3) using the genus of specific primers JDP1 (5'- GGCCAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGCTAGGGGAGTCA-3') [32].

The amplifications were performed in a final volume of 50µL containing 25µL Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark), 5 µL template DNA, 0.1µM of each primers and 17.5 µL distilled water. The thermal cycling (Corbet research, Australia) conditions began with an initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of denaturing for 30 seconds at 94˚C, annealing for 45 seconds at 56˚C, the extension took for 30 seconds at 72˚C, and the final elongation was done for 10 min at 72˚C. The PCR products were electrophoresed on a 2% agarose gel, and then stained with ethidium bromide and visualized under a UV Trans illuminator.

3. Results and Discussion

3.1. Morphological identification

The recognition of *Acanthamoeba* at the genus level in the survey was based on the double walled cysts (Fig. 1) and the flattened trophozoites with acanthapodia, spine-like pseudopodia, on their surfaces (Fig.2). Out of 60 water samples collected in different recreational water sources, 30 (50%) were positive for *Acanthamoeba* spp based on the morphological criteria in non-nutrient agar (NNA) culture. The dams were the water sources with the highest number of positive samples, displaying 10/12 (83%) of positive culture isolates. A lower rate of positivity with 2/14 (14.3%) was obtained from channels (Table 1).

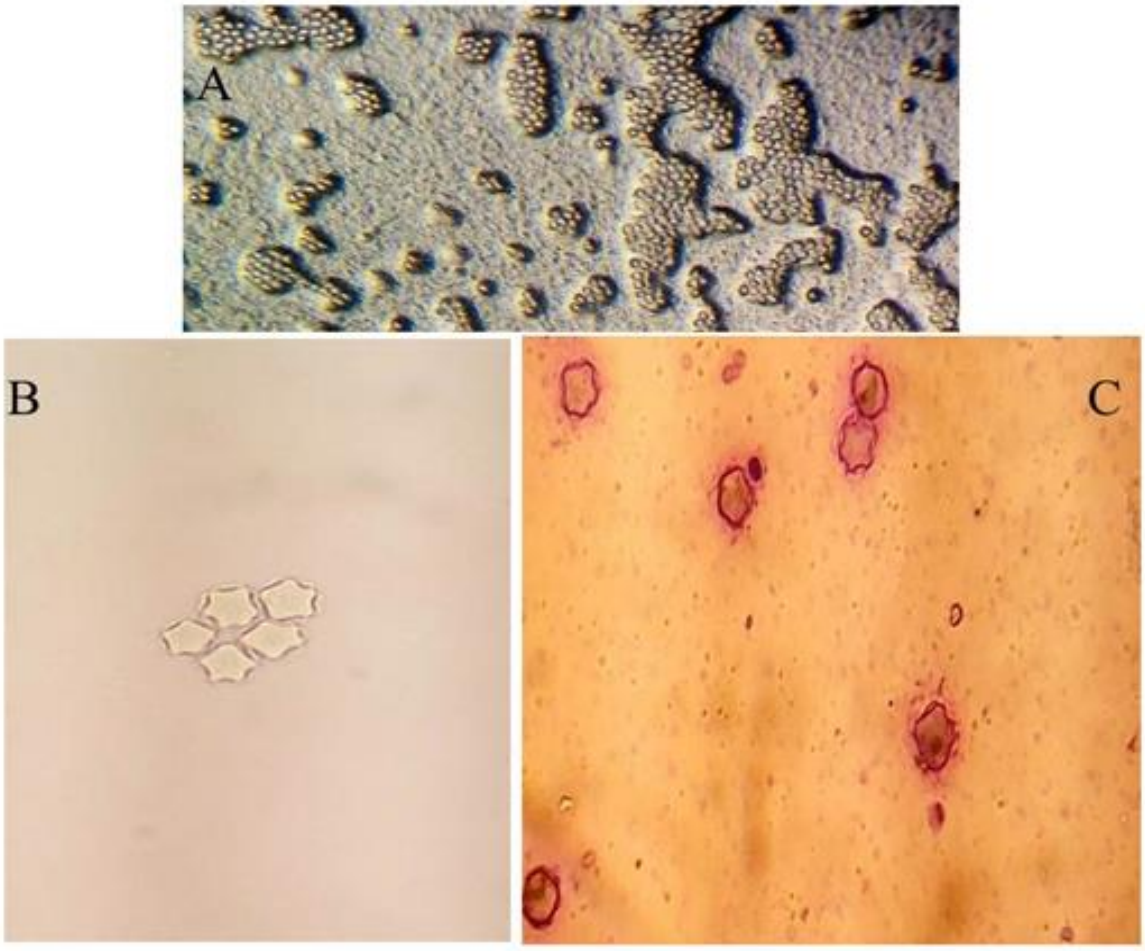

Fig. 1: Light micrographs of *Acanthamoeba* spp. cysts obtained from water samples.

A: Cysts on NNA (10X), B: Cysts in saline (40X), C: Cysts in Giemsa stain (40X).

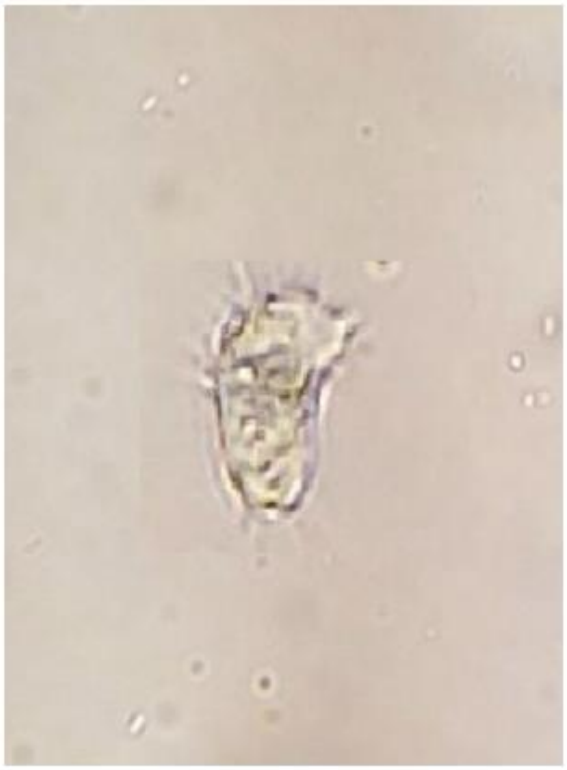

Fig. 2: *Acanthamoeba* trophozoites in saline (40X).

Table 1: The Frequency of *Acanthamoeba* spp. in various water sources in Zanjan province, Iran.

Water sources	No. of examined samples	Positive Culture samples (%)
Park	10	5(50%)
Square and fountain	24	13 (54.2%)
Dam	12	10 (83.32%)
Channel	14	2 (14.3%)
Total	60	30 (50%)

3.2. The Molecular identification

The cultured positive samples of *Acanthamoeba* isolates, were further confirmed by the polymerase chain reaction (PCR) method, which were done by amplifying a portion of the 18S rRNA gene applying the genus specific primers pairs, The JDP1 and JDP2, and a nearly 500bp band detected on the agarose gel for all the positive isolates (Fig. 3).

The genus of *Acanthamoeba*, is the etiological agent of granulomatous amoebic encephalitis (GAE), The *Acanthamoeba* keratitis (AK), and the disseminated tissue infections, are the ubiquitous protozoan parasite which have been widely found in the various water sources such as lakes, pools, tap water, thermal water, bottled mineral waters, cooling water, sea water, treated water, aquarium, rain water, wastewater and recreational waters [17, 33-36].

The food availability for the growth of *Acanthamoeba* spp and the resistance of the cyst stage of these amoebae to chlorination, ozonation and filtration by biofilm formation, probably creates the potential for high distribution of *Acanthamoeba* spp in the aquatic environments (25, 37-39). Thus, this alarmingly frequency of this amoeba in water resources represents a significant hazard for the high risk populations, including the contact lens wearers and immunocompromised individuals [13, 21, 26].

There are a few reports available regarding the presence of *Acanthamoeba* spp in different water sources in Iran [12, 13, 15, 22, 24, 40]. The results of previous studies in Iran showed that an increasing rate, in addition to being in the Acanthodea keratitis, was also in an undistinguished encephalitis in the immunocompromised patients [5, 19, 20, 22].

Accordingly, the isolation and identification of *Acanthamoeba* spp in water sources may play an important role in the prevention and control of the above mentioned serious problems. This is the first comprehensive research representing the occurrence of *Acanthamoeba* spp, using the morphological and molecular methods, in Zanjan province, Iran. This province is located in North-West of Iran bordering the provinces of East Azerbaijan, West Azerbaijan, Hamedan, Kordestan, Gilan, Ghazvin and Ardabil. Zanjan covers a region of 21.773km² where there are a lot of water sources such as dams, rivers, mineral water springs and green areas with superficial waters which are a hygienic risk for the populations who use these kinds of water in their life. In this survey, based on the morphological assessment, about half of the water samples were identified as the *Acanthamoeba* spp. Also, all of

the positive samples were reconfirmed by the molecular method as *Acanthamoeba* spp, the obtained results in the present study give further evidence of the presence of *Acanthamoeba* spp in the surface waters exist in Iran which is in accordance with other investigations worldwide [6, 15, 17, 22, 41-43].

The majority of the studies which studied the water samples were associated with the human recreational activity .

Therefore, the water sources in Zanjan province could be considered a significant public health hazard. The rate of contamination in Zanjan province was lower than it is in other studies which have been carried out in worldwide such as Khyber Pakhtunkhwa/Pakistan (92%), Northern parts/Poland (65%), Mazandaran/Iran (85%), Kish Island/Iran (66.7%), Bojnurd/Iran (68%), Ahvaz/Iran (71.6%) and Tehran/Iran (80%) [12, 13, 25, 40, 44-46]. The level of water contamination detected in this research was higher than the reports from Central and Southern parts/Italy (28.7%), Kayseri/Turkey (6.5%), Sivas/Turkey (4.4%), Leon/Nicaragua (21%), Birjand/Iran (38%), East Azerbaijan/Iran (25.4%), Gilan/Iran (30%), Shiraz/Iran (32.5%) and Tehran/Iran (32%) [5, 24, 36, 47-52]. Overall, the high variability observed in the frequency rates in different localities could be related to several parameters, including the geographical factors, the climatic conditions, the seasonal changes, the water type, the samples of collection, the methodology of study and the diagnostic techniques [9, 40, 52, 53]. In the present work, the highest distribution of *Acanthamoeba* spp were found in dams and water sources, while the lowest frequency was recorded in the channels of water resources. The discrepancy of contamination rate in various kinds of aquatic environments may be due to the number of water samples examined, the water exposure through air, soil and feces, the flow and circulation of waters, water speed, the rate of water temperature , the biotic and abiotic factors, the existence of phagocytosing microorganisms and water treatment condition.

Thus, the comparison of all such investigations is not logical [15, 17, 38, 40].

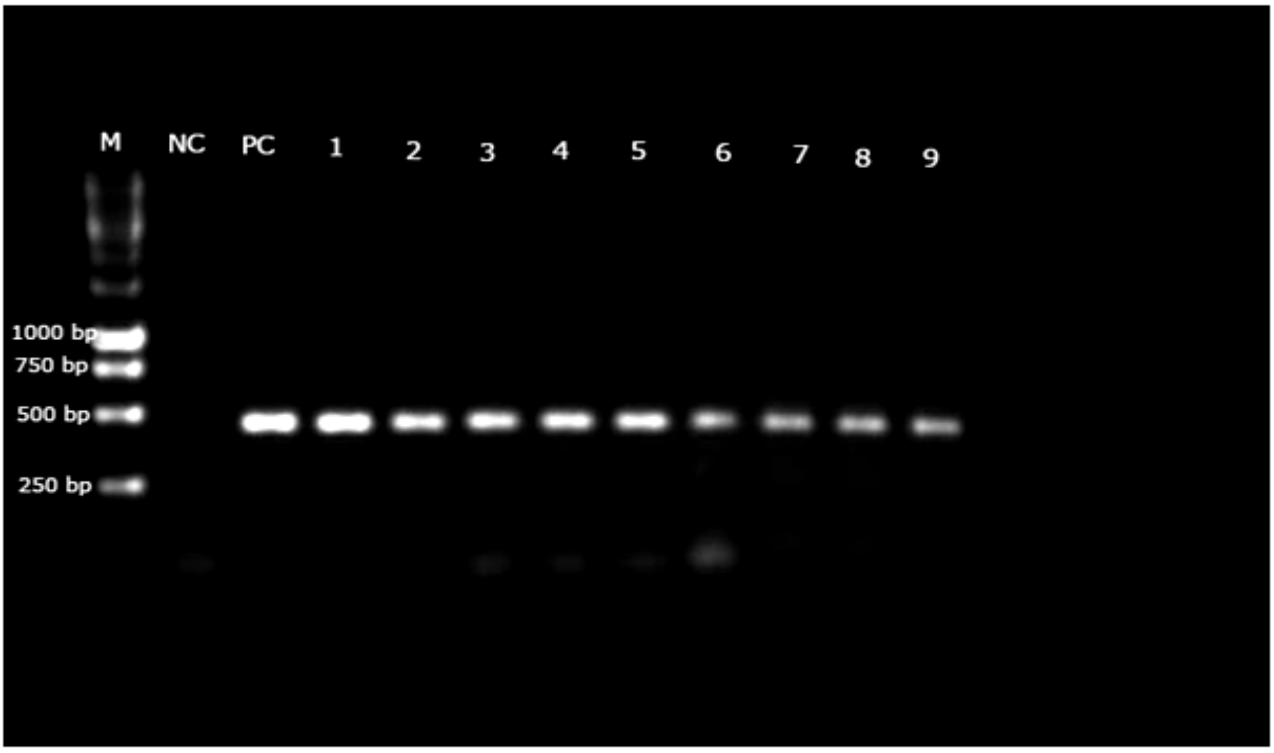

Fig. 3: The Gel electrophoresis of the PCR products of *Acanthamoeba* spp. isolated from the water samples of Zanjan province, Iran. Lane M: standard DNA marker (250 bp), lane NC: negative control, lane PC: positive control and the lanes 1 to 9 are representative of the water *Acanthamoeba* isolates.

4. Conclusion

Although, the present survey gives the baseline information about water contamination with *Acanthamoeba* spp and will provide important knowledge regarding that water could be a significant transmission agent for *Acanthamoeb* spp, it requires further studies to focus on the determination of *Acanthamoeba* genotypes in the environmental samples and human in order to develop the proper methods for prevention and control of the severe and fatal complications.

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